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Yeast acetic acid-induced programmed cell death can occur without cytochrome *c* release which requires metacaspase YCA1

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ABSTRACT

To investigate the role of cytochrome *c* (cyt *c*) release in yeast acetic acid-induced programmed cell death (AA-PCD), wild type (wt) and cells lacking metacaspase (Δyca1), cytochrome *c* (Δcyc1,7) and both (Δcyc1,7Δyca1) were compared for AA-PCD occurrence, hydrogen peroxide (H_2O_2) production and caspase activity. AA-PCD occurs in Δcyc1,7 and Δcyc1,7Δyca1 cells slower than in wt, but similar to that in Δyca1 cells, in which no cytochrome *c* release occurs. Both H_2O_2 production and caspase activation occur in these cells with early and extra-activation in Δcyc1,7 cells. We conclude that alternative death pathways can be activated in yeast AA-PCD, one dependent on cyt *c* release, which requires YCA1, and the other(s) independent on it.

1. Introduction

In order both to accelerate the understanding of mammalian apoptosis and to discover new pharmacological target molecules for apoptosis control, use of yeast is advisable [1,2]. Yeast allow for both over-expression and knockout of certain genes involved in cell death thus offering additional tools to elucidate how cell death occurs. In particular, *Saccharomyces cerevisiae* can undergo cell death exhibiting specific hallmarks of apoptosis such as DNA fragmentation, which itself was also found in other forms of programmed cell death (PCD) [3,4]. PCD takes place as a result of a variety of stimuli, including treatment with hydrogen peroxide (H_2O_2), hyperosmotic stress, pheromone, acetic acid (AA) [5]. In particular, acetic acid-induced PCD (AA-PCD) in *S. cerevisiae*, first shown in [6], has been investigated in some detail as a function of time: reactive oxygen species (ROS) production and release of cytochrome *c* (cyt *c*) to the cytosol take place and H_2O_2 is a trigger for AA-PCD [7–10]. Yeast cells lacking the metacaspase-encoding gene YCA1 (Δyca1) show a reduced rate of death, YCA1 contributing to AA-PCD not only in a manner related to caspase-like activity [11].

The release of cyt *c* is a PCD feature shared among mammalian, plant and yeast mitochondria [12–15]. In this regard, we have shown that the released cyt *c* works both as a ROS scavenger and as a respiratory substrate both in cerebellar granule [16] and in yeast [9] cells. However whether cyt *c* release is the essential step in AA-PCD as well as the YCA1 role in cyt *c* release remain to be established [17–19]. Thus we compared wild type (wt) and Δyca1 cells with the respective mutant cells lacking cyt *c* isoforms for the occurrence of AA-PCD, H_2O_2 level and caspase activation. We show that AA-PCD can occur without cyt *c* release, which itself requires YCA1.

2. Materials and methods

2.1. Strains, media and growth conditions

The *S. cerevisiae* strains used were W303-1B (*MATα ade2leu2-his3trp1ura3*), Δyca1 (W303-1B*yca1Δ::KanMX4*) [11], Δcyc1,7 (W303-1B*cyc1Δ::URA3cyc7Δ::TRP1*) [20], which was kindly provided by Alexander Tzagoloff, Δcyc1,7Δyca1 (W303-1B *cyc1Δ::URA3cyc7Δ::TRP1yca1Δ::KanMX4*). The last strain was constructed by replacing YCA1 gene with Δyca1::KanMX4 cassette in Δcyc1,7 cells as in [11]. In all cases, cells were grown at 30 °C in rich medium (1% yeast extract and 2% Bacto-peptone) containing 2% dextrose (YPD).

Abbreviations: AA, acetic acid; PCD, programmed cell death; AA-PCD, acetic acid-induced programmed cell death; wt, wild type; Δyca1, YCA1-lacking; Δcyc1,7, cytochrome *c* lacking; H_2O_2, hydrogen peroxide; cyt *c*, cytochrome *c*; DCF, dichlorofluorescein diacetate; z-VAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-fluoromethylketone

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Δyca1 cells were transformed with recombinant pRS416 plasmid containing enhanced green fluorescence protein (eGFP) fused to the 3′ end of YCA1 coding sequence under control of MET17 inducible promoter (kindly provided by Michael Duszenko) [21]. Δyca1-YCA1eGFP cells were grown in selective medium containing 0.67% yeast nitrogen base with ammonium sulphate, 2% dextrose and 0.2% drop-out mix (US Biological, MA, USA) without uracile and methionine, for plasmid selection and promoter induction, respectively.

2.2. AA-treatment and cell viability measurement

The former was performed as in [6] and the latter was determined by measuring colony-forming units (cfu) after 2 days of growth on YPD plates at 30 °C.

2.3. TUNEL assay and intracellular H_2O_2 detection

To detect DNA fragmentation we used TUNEL assay. Control and AA-treated cells (10^8) were harvested at different times. TUNEL assay was performed as in [9]. Intracellular H_2O_2 was detected with the fluorophore 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes) as in [10].

2.4. Immunoblotting

Cytosolic and mitochondrial fractions were isolated from control and AA-PCD Δyca1 cells and immunoblot analysis were performed as described in [9]. Polyclonal anti-cytochrome *c* was kindly provided by Fred Sherman (University of Rochester Medical Center, Rochester, NY, USA).

2.5. Caspase activity assay

Caspase activity was measured as in [22]. Control and AA-treated cells were harvested at different times, washed with Tris–HCl (pH 7.2) and resuspended in 200 µl lysis buffer, containing 20 mM Hepes–NaOH (pH 7.3), 0.5% Nonidet P40, 84 mM KCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 1 µg ml^{-1} leupeptin and 1 mM PMSF. Cells were lysed with ice-chilled glass beads (0.5 mm) alternating 30 s on vortex and 30 s on ice. Cell lysates were centrifuged at 20 000×*g* for 30 min and supernatants were used for the assay. Fifty microgram protein was incubated at 37 °C either with 40 µM N-acetyl-Ile-Glu-Thr-Asp-7-amino-4-methyl-coumarin (Ac-IETD-AMC), the mammalian caspase-8 substrate, or N-acetyl-Val-Glu-Ile-Asp-7-amino-4-methyl-coumarin (Ac-VEID-AMC), caspase-6 substrate (Alexis Biochemicals) in 50 mM Hepes–NaOH (pH 7.3) containing 100 mM NaCl, 10% sucrose, 0.1% CHAPS and 10 mM DTT. Release of AMC was detected using an LS50 Perkin–Elmer spectrofluorimeter (excitation of 370 nm and emission of 440 nm). All the values were corrected for the intrinsic substrate fluorescence. Protein concentration was determined according to Bradford assay [23].

3. Results

3.1. The occurrence of AA-PCD in Δcyc1,7 and Δcyc1,7Δyca1 cells

We have already shown that wt and Δyca1 yeast cells can undergo AA-PCD in a similar manner [11]. Thus to ascertain whether cyt *c* release is crucial for AA-PCD to occur, we used Δcyc1,7 and Δcyc1,7Δyca1 cells, each lacking the two cyt *c* isoforms encoded by the nuclear genes CYC1 and CYC7 [20], and derived from wt and Δyca1 cells, respectively. First the viability in wt, Δyca1, Δcyc1,7 and Δcyc1,7Δyca1 cells up to 200 min after AA-PCD induction was investigated (Fig. 1A). A progressive decrease in cell viability was observed in all cases, with a percentage of survival of about 80% after 60 min, for the three mutants, and about 40% for wt. At 200 min after PCD induction 15% survival was found in all mutant cells, compared with 2% survival for wt (Fig. 1A). The specific AA-PCD rates were virtually the same for the mutant strains, but significantly lower than that calculated in wt cells (inset to Fig. 1A), as judged by statistical analysis (Student's t-test, $P < 0.01$).

To find out whether AA-induced death in Δcyc1,7 and Δcyc1,7-Δyca1 cells takes place with DNA fragmentation, TUNEL assay was carried out in cells *en route* to death with wt and Δyca1 cells used as positive controls [9,11] (Fig. 1B). Both AA-treated Δcyc1,7 and Δcyc1,7Δyca1 cells showed TUNEL-positive nuclei similarly to wt and Δyca1, with the percentage increase up to 40% at 150 min in both cases (only 2% in control cells) (Fig. 1B).

3.2. AA-PCD in Δyca1 cells occurs without cytochrome c release

Having established that AA-PCD in Δcyc1,7 and Δcyc1,7Δyca1 cells takes place with similar time courses and with DNA strand brakes occurring in any case, to ascertain whether YCA1 deletion itself could affect cyt *c* release we assayed immunologically the

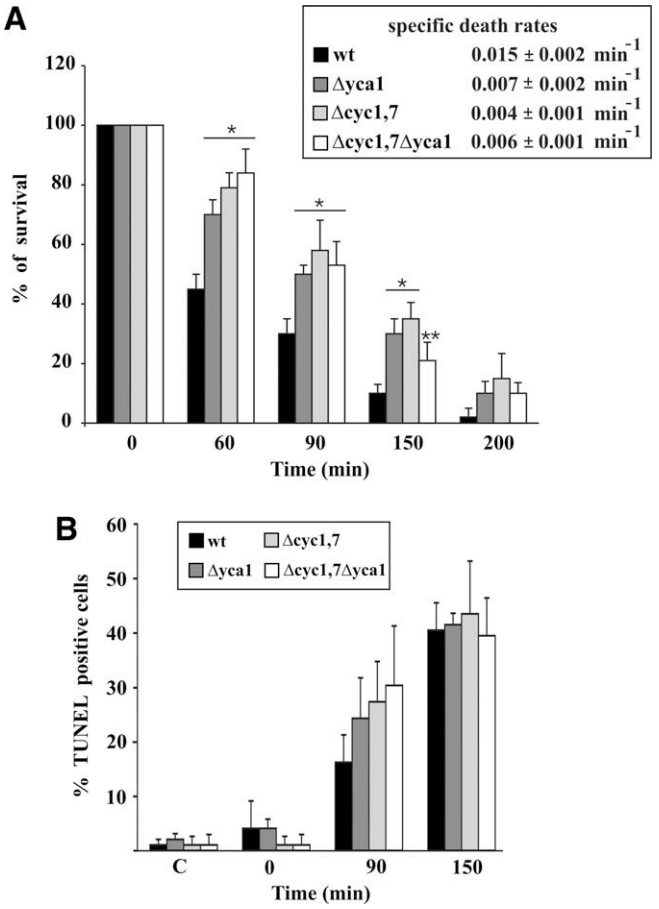

Fig. 1. Occurrence of AA-PCD in wt, Δyca1, Δcyc1,7 and Δcyc1,7Δyca1 cells. (A) wt, Δyca1, Δcyc1,7 and Δcyc1,7Δyca1 were treated with 80 mM AA. Cell viability was analyzed at indicated times (A) by measuring colony-forming units (cfu) after 2 days of growth at 30 °C. Cell survival (100%) corresponds to the cfu at time zero. The means of four independent experiments with standard deviations are reported. $^{*}P < 0.01$; $^{**}P < 0.05$, as compared to wt cells, Student *t*-test. Death rates were calculated as the slope of the linear part of the semi-logarithmic plot of the number of cfu as a function of time. DNA strand breaks were detected by using the TUNEL assay. (B) Percentage of TUNEL-positive control (C) and AA-treated cells at indicated times. At least 400 cells were evaluated for each sample and for each time analyzed in two independent experiments.

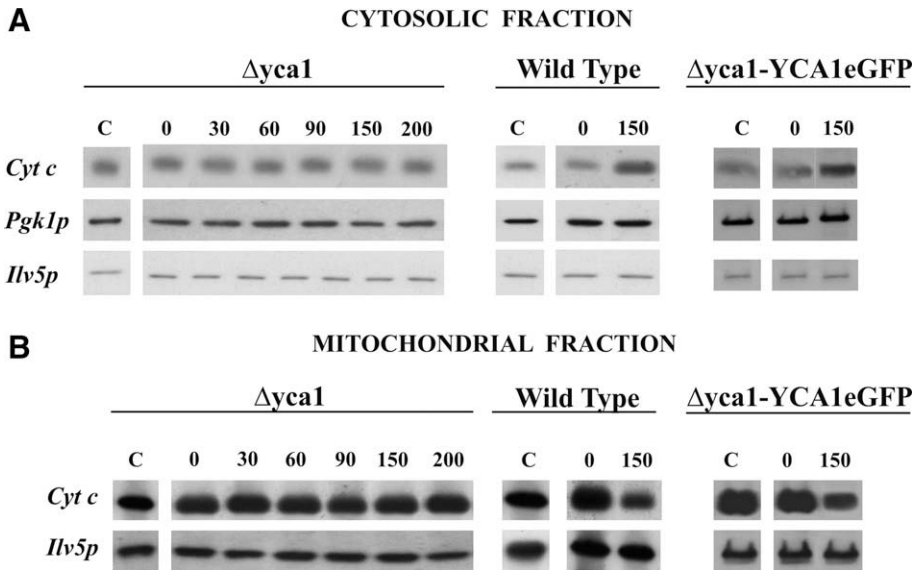

Fig. 2. Western blot analysis of cytochrome *c* in AA-PCD Δyca1 cells. Representative immunoblots of cyt *c*, Pgk1p and Ilv5p are reported. (A) Cytosolic and (B) Mitochondrial fractions from control (C) and AA-PCD cells were obtained at indicated times and analyzed by western blot analysis as described in Section 2. Antibodies against Pgk1p and Ilv5p, were used to normalize the protein amount loaded onto the gel.

amount of cyt *c* in cytosolic and mitochondrial fractions isolated at different times from control and AA-treated Δyca1 cells by using a polyclonal anti-cytochrome *c* antibody (Fig. 2). As a loading control use was made of two monoclonal antibodies against Pgk1p and Ilv5p, cytosolic and mitochondrial matrix protein markers, respectively (Fig. 2A). A low but constant amount of Ilv5p was found in the cytosolic fraction of both control and AA-PCD cells up to 200 min (Fig. 2A), resulting from mitochondrial damage during the isolation procedure as already observed in [9]. In distinction with wt cells in which at 150 min after AA-treatment the cytosolic cyt *c* amount increases up to 140% of the control as in [9], no cyt *c* release was found in Δyca1 cells. Consistently no change in the mitochondrial cyt *c* amount was found in Δyca1 cells, as opposed to wt cells in which it decreased (Fig. 2B). Since Δyca1 cells were shown to accumulate deleterious mutations with time [24], we tried to ascertain whether YCA1 deletion *per se* is responsible for the lack of cyt *c* release. Thus, we over-expressed in Δyca1 cells a YCA1-eGFP fusion gene under the control of MET17 inducible promoter [21], and induced cell death with AA. We assayed immunologically the amount of cyt *c* in cytosolic and mitochondrial fractions isolated from AA-treated Δyca1-YCA1eGFP cells. Similarly to wt cells a higher cyt *c* level was found in the cytosol with respect to control untreated cells after 150 min (Fig. 2A). Consistently, a decrease in mitochondrial cyt *c* was found in AA-treated Δyca1-YCA1eGFP cells at the same time (Fig. 2B). These data demonstrate that the lack of the cyt *c* release is dependent on YCA1.

3.3. H_2O_2 level and caspase activity in wt, Δyca1, Δcyc1,7 and Δcyc1,7Δyca1 cells en route to AA-PCD

We have already shown that in wt cells undergoing AA-PCD, H_2O_2 production and caspase activation take place before (15 min) and after (200 min) cyt *c* release, respectively [8,9,11].

In Δyca1, Δcyc1,7 and Δcyc1,7Δyca1 cells, H_2O_2 levels were analyzed as a function of time by using H_2DCF-DA [8,10] (Fig. 3). As a result of AA-treatment, but not in control cells, H_2O_2 was detectable in the first 60 min in all cell types: while very low level of H_2O_2 is measured in control cells, a burst of H_2O_2 was observed in all mutant strains and in wt at 15 min, with about 60% of DCF-positive cells. A decrease in H_2O_2 level was found up to 90 min.

Caspase-like activity in cell-free extracts was measured fluorimetrically by using the mammalian caspase substrates Ac-IETD-AMC and Ac-VEID, efficiently cleaved also in yeast [22,25]. In particular at 150 min, in Δcyc1,7 cells both the investigated caspase activities were significantly higher than those in the other cells ($P < 0.001$) (Fig. 4). In both cases the caspase activities, which are low and constant in control cells, progressively increased up to 200 min in AA-PCD wt, Δyca1, Δcyc1,7 and Δcyc1,7Δyca1 cells (Fig. 4). In particular, 200 min after PCD induction, the IETD-ase activity increased of about 5-, 8-, 17-fold in Δyca1, wt and Δcyc1,7Δyca1, Δcyc1,7 cells, respectively, over control (Fig. 4A). At the same time, the VEID-ase activity increase was about 3-fold in Δyca1, 6-fold in wt, 12-fold in Δcyc1,7Δyca1 and 17-fold in Δcyc1,7 cells over control (Fig. 4B). We confirmed that both the

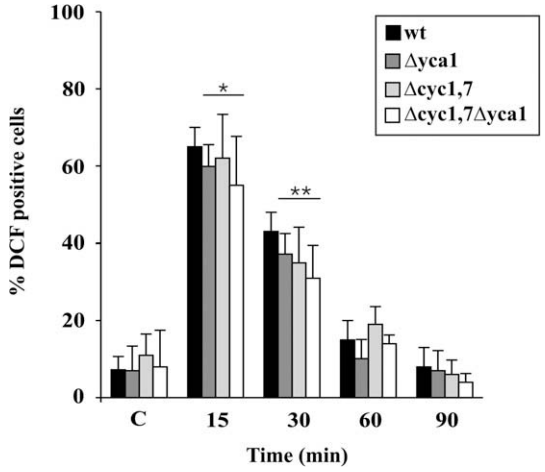

Fig. 3. H_2O_2 levels in Δyca1, Δcyc1,7 and Δcyc1,7Δyca1 cells *en route* to AA-PCD. Cells were incubated with H_2DCF-DA in the absence or in the presence of AA. Cells were collected at indicated times and H_2O_2 was detected by fluorescence microscopy as described in Section 2. Percentage of DCF-positive control (C) and AA-treated cells at indicated times: wt, Δyca1, Δcyc1,7 and Δcyc1,7Δyca1. At least 400 cells were evaluated for each sample and for each time analyzed in two independent experiments. $^{*}P < 0.001$; $^{**}P < 0.01$, as compared to respective control (C) cells, Student *t*-test.

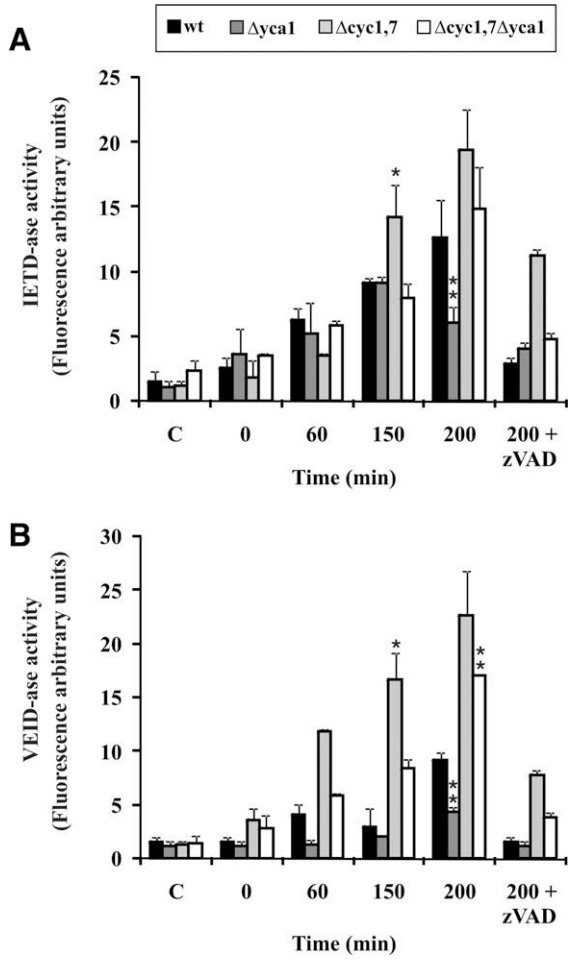

Fig. 4. Caspase activity in wt, Δyca1, Δcyc1,7 and Δcyc1,7Δyca1 cells *en route* to AA-PCD. Cell-free extracts were prepared at indicated times from control (C) and AA-treated wt, Δyca1, Δcyc1,7 and Δcyc1,7Δyca1 cells as described in Section 2. Extracts were tested for their ability to cleave the fluorogenic caspase substrates Ac-IETD-AMC (A) and Ac-VEID-AMC (B). AA-PCD cell extracts (200 min) were pre-incubated with z-VAD-fmk (100 μM) for 5 min at room temperature before assay. Activities are reported as fluorescence arbitrary units. $^{*}P < 0.001$ when comparing Δcyc1,7 *versus* wt, Δyca1 and Δcyc1,7Δyca1, $^{**}P < 0.01$ when comparing Δyca1 *versus* wt and Δcyc1,7Δyca1 *versus* Δcyc1,7 cells; Student *t*-test.

caspase-like activities *en route* to AA-PCD were inhibited by the pan-caspase inhibitor z-VAD-fmk.

4. Discussion

Yeast AA-PCD has been shown to occur with a partial inhibition of cyt *c* release [26]. We show here that cell death can occur, even if at a lower rate, without cyt *c* release in AA-treated *S. cerevisiae* [Fig. 1], thus raising some questions as to the role of the released cyt *c* in PCD. Indeed TUNEL assay, commonly used to identify death via apoptosis, detects also necrotic death [3,4], however we propose that mutant cells die in a manner distinct to the wt in the light of Fig. 1 in which in spite of similar percentage of TUNEL-positive cells, the survival of mutant cells is higher. In the light of Fig. 1, in which we show that specific death rate of cells lacking cyt *c* is significantly lower than that of wt cells, we conclude that cyt *c* participates to AA-PCD, in agreement with the relevance of mitochondrial functionality in this process [7,9,26], although its role seems dispensable for PCD to occur. Further investigation is required to study whether and how mitochondria play a role in cyt *c*-independent AA-PCD pathway.

We show that YCA1 is strictly required for cyt *c* release to occur [Fig. 2], perhaps in addition to the H_2O_2 production, which takes place in Δyca1 cells [Fig. 3], but is apparently ineffective in releasing cyt *c per se*. This could be either a direct effect of YCA1 deletion or the result of deleterious mutations accumulated with time [24]; the latter hypothesis can be ruled out since the introduction in Δyca1 cells of a functional copy of YCA1 under control of an inducible promoter causes cyt *c* release.

A further role for YCA1 is to favor caspase activation, as shown in Fig. 4 in which a reduced caspase activity is measured in Δyca1 cells with respect to wt cells. This could be due to the absence of released cyt *c* in these cells according to [27]. We show that YCA1-dependent as well as YCA1-independent caspase-like proteases are involved in AA-PCD. Even though the proteases responsible for the cleavage of mammalian caspase substrates in yeast are actually unidentified, we can exclude that they include Kex1, that is unable to cleave VEID substrate [28]. Fig. 4 raises a question as to the role of the released cyt *c* in caspase activation. The reason why deletion of both the cyt *c* isoforms results in early and extra caspase activation must remain a matter of speculation; we might suggest that released cyt *c* regulates caspase activation, which is assumed to be somehow inhibited by the released cyt c (Fig. 4).

Altogether these data support the hypothesis that compensatory and alternative pathways can be activated in AA-PCD, when the main route triggered by the death stimulus is blocked in certain steps, for instance cyt *c* release.

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